

# Activation-Induced Cytidine Deaminase (AID) Deficiency Causes the Autosomal Recessive Form of the Hyper-IgM Syndrome (HIGM2)

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HIGM2 patients (and in AID<sup>-/-</sup> mice) demonstrates the absolute requirement for AID in several crucial steps of B cell terminal differentiation necessary for efficient antibody responses.

## Introduction

Production of highly efficient neutralizing antibodies requires affinity maturation of antibody responses. This crucial event takes place within lymphoid organs in germinal centers (GC) and is T cell driven (Liu et al., 1989; Berek et al., 1991; Kuppers et al., 1993). Four major events occur, i.e., B cell proliferation, generation of somatic hypermutations in the immunoglobulin (Ig) variable region (V) genes able to increase the affinity of antibodies for antigens, positive selection of B cells bearing a B cell receptor (BCR) of high affinity for antigen, and Ig class switch recombination (CSR) toward the production of IgG, IgA, and IgE (Rajewsky, 1996). The molecular mechanisms involved in these events are not precisely delineated.

A cognate interaction between CD40-ligand (CD40L or CD154) expressed on activated helper T cells and CD40 constitutively expressed on B cells is required for B cell terminal maturation. Cross-linking of CD40 molecules leads to B cell proliferation, rescue of B cells from apoptosis, somatic hypermutations of Ig V genes, and CSR (Clark and Ledbetter, 1986; Rousset et al., 1991; Lederman et al., 1992; Spriggs et al., 1992; Zan et al., 1999). This activation pathway requires adaptor proteins, such as the members of the TRAF family (Hu et al., 1994; Cheng et al., 1995; Sato et al., 1995; Ishida et al., 1996a, 1996b) or BLNK (Jumaa et al., 1999; Hayashi et al., 2000) and induces phosphorylation of tyrosine kinases (Ren et al., 1994) and activation of transcription factors, such as NF- $\kappa$ B (Berberich et al., 1994). However, none of these molecules have been shown to be directly involved in the generation of somatic mutations of Ig V genes or CSR. Recently, the Activation Induced cytidine Deaminase (AID) protein has been identified in the mouse (Muramatsu et al., 1999). AID expression is strictly restricted to germinal center B cells and B cells induced to switch in vitro in the presence of LPS or soluble CD40L and IL-4, suggesting a role for AID in terminal B cell differentiation. AID is a novel member of the RNA-editing deaminase family, which contains proteins able to create new functional products from mRNA by base substitution (Scott, 1995; Simpson et al., 1995). An increasing number of human genes are known to be edited. The most widely studied is the mRNA editing of apolipoprotein B, induced by the cytidine deaminase APOBEC-1 (Navaratnam et al., 1993; Teng et al., 1993) which is homologous to AID.

A rare human immunodeficiency, the Hyper-IgM syndrome (HIGM), is characterized by normal or elevated serum IgM levels with absence of IgG, IgA, and IgE, resulting in a profound susceptibility to bacterial infections (Notarangelo et al., 1992). The molecular basis of the X-linked form (HIGM1) is due to mutations in the gene coding for CD40L (Allen et al., 1993; Aruffo et al., 1993; Di Santo et al., 1993; Fuleihan et al., 1993; Korthauer et al., 1993). Besides the Ig switch defect, these

## Summary

The *activation-induced cytidine deaminase (AID)* gene, specifically expressed in germinal center B cells in mice, is a member of the cytidine deaminase family. We herein report mutations in the human counterpart of *AID* in patients with the autosomal recessive form of hyper-IgM syndrome (HIGM2). Three major abnormalities characterize AID deficiency: (1) the absence of immunoglobulin class switch recombination, (2) the lack of immunoglobulin somatic hypermutations, and (3) lymph node hyperplasia caused by the presence of giant germinal centers. The phenotype observed in

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patients exhibit a remarkable defect in germinal center formation (Facchetti et al., 1995). These observations emphasize the role of CD40L/CD40 interaction in the terminal differentiation of B cells in secondary lymphoid organs. In these patients, B cells are intrinsically normal since they can be normally triggered by CD40 agonists to CSR in vitro (Allen et al., 1993; Aruffo et al., 1993; Durandy et al., 1993; Korthauer et al., 1993).

Another HIGM syndrome with autosomal recessive inheritance (HIGM2) has been described (Callard et al., 1994; Conley et al., 1994). In these patients, CD40L gene sequencing and membrane molecule expression are normal and, in contrast to CD40L-deficient patients, B cells do not undergo CSR in vitro in the presence of CD40-agonists. CD40 gene sequence and CD40 molecule expression are normal in these patients (Durandy et al., 1997). A defect of other molecules involved in CD40 signaling such as the TRAF proteins (Hu et al., 1994; Cheng et al., 1995; Sato et al., 1995; Ishida et al., 1996a, 1996b) or proteins involved in the activation of transcription factor NF- $\kappa$ B such as TANK (Cheng and Baltimore, 1996; Rothe et al., 1996) or NIK (Malinin et al., 1997; Song et al., 1997) have also been excluded (unpublished data). In addition, activation by CD40 agonists of monocytes and dendritic cells from these patients is consistently detectable (Revy et al., 1998), strongly suggesting that the intrinsic defect in HIGM2 patients originates in B cells, either in a B cell-specific CD40 triggered event, or in the CSR mechanism itself.

In order to identify the genetic basis of the HIGM2 syndrome, we performed a genome-wide search for susceptibility loci using polymorphic microsatellite markers in consanguineous families. We demonstrated a strong linkage to chromosome 12p13. As the human *AID* (*huAID*) gene, a possible candidate gene, also maps to 12p13 (Muto et al., 2000), we investigated whether human *AID* gene defects could cause the HIGM2 syndrome. We describe 10 independent mutations in the *huAID* gene in 18 patients with HIGM2 from 12 families. We demonstrate that the HIGM2 syndrome is characterized by defective CSR, defective generation of somatic mutations of the Ig V genes, and by abnormal germinal centers, showing that *AID* gene expression plays a key role in terminal B cell differentiation.

## Results

### Patients' Characteristics

Eighteen patients from 12 unrelated families (Figure 1) were diagnosed with hyper-IgM syndrome (HIGM) defined by markedly diminished serum levels of IgG and IgA with a normal or increased serum level of IgM (Table 1). All patients presented in childhood with recurrent bacterial sino-respiratory and gastro-intestinal tract infections (Table 1). A characteristic feature observed in most of the patients (13/18) was lymphoid hyperplasia, leading to tonsillectomy in 5 of them. No opportunistic infections occurred, in contrast to those patients with HIGM1 (Levy et al., 1997). Anti-tetanus IgG antibodies were not found despite immunization (data not shown), while IgM isohemagglutinins, when evaluable, were detected (Table 1). From diagnosis, all patients were treated with intravenous Ig substitution. As shown in Figure 1, both males ( $n = 13$ ) and females ( $n = 5$ ) were affected. Family consanguinity was frequent (9 out of 12), suggesting an autosomal recessive inheritance pattern. HIGM1 was excluded in all, since CD40L gene

sequencing was normal and CD40L was normally expressed on activated T cells. CD40 gene sequence and/or membrane expression revealed no abnormality. Peripheral blood B cell (CD19<sup>+</sup>) counts were normal. All B cells expressed sIgM and sIgD while some CD19<sup>+</sup> B cells from age-matched controls did not express either sIgM or sIgD (data not shown). CD27, a recently identified marker of memory B cells (Klein et al., 1998) was expressed on a normal proportion (from 9% to 45%) and with the same intensity on B cells, as compared with age-matched controls (Table 2 and data not shown).

In vitro activation of peripheral blood mononuclear cells (PBMC) by soluble CD40L (sCD40L) and IL-4, which regularly induced IgE production by B cells from controls and CD40L-deficient patients (Durandy et al., 1993), was ineffective in the 15 tested HIGM2 patients (Table 2). However, in the same culture conditions,  $\text{I}\epsilon\text{-C}\epsilon$  sterile transcripts were inducible (Figure 3C). T cell (CD3<sup>+</sup>) and T cell subset numbers (CD4<sup>+</sup> and CD8<sup>+</sup>) as well as in vitro T cell proliferation to mitogens and antigens was normal (data not shown).

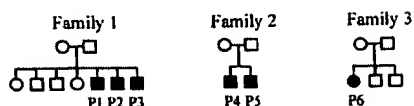
### Identification of the HIGM2 Genetic Locus

Genetic mapping was investigated by studying the segregation of polymorphic microsatellite markers in 10 families (8 consanguineous). The genome was randomly screened by using 280 polymorphic markers. We performed homozygosity mapping in the eight consanguineous families and performed a bipoint LOD score analysis in all affected families. Disease segregation was compatible with the *D12S372* marker (telomeric region of short arm of chromosome 12) in the studied families. Further analysis with additional markers was performed and two additional multiplex nonconsanguineous families were included. Multipoint analysis with those additional markers gave a maximum LOD score of 10.45 for marker *D12S1695* ( $\theta = 0.00$ ). Recombination analysis defined the critical genetic interval on 12p13, as no recombination was found in all tested families between markers *D12S397* and *D12S1697*, encompassing a 4.5 cM region. Among a YAC contig covering the delineated region, the 943a9 YAC, which contains both *D12S1695* and *D12S336* markers, was shown to harbor exon 2 of *huAID* (Figure 2A).

### Sequencing of *huAID* Gene in HIGM2 Patients

Because the expression of mouse *AID* is strictly restricted to GC B cells and B cells induced to switch in vitro, and the *huAID* gene (Muto et al., 2000) and HIGM2 locus colocalize on chromosome 12p13, we studied *huAID* as a possible candidate gene. The coding sequence of *huAID* gene, including the 5 exons and the adjacent intronic regions, was sequenced in 18 patients. Ten independent mutations were found (Figures 2B and 2C). Five homozygous missense mutations, causing different amino acid substitutions, were identified in 7 consanguineous families. The same mutation was observed in exon 2 in patients P7, P11-P12, and P18 from 3 unrelated families of Turkish origin. Segregation of surrounding polymorphic markers clearly indicate that two of 3 families (families 4 and 7) have received the same alleles, suggesting a common origin of the mutation. In three other Turkish families, three different point mutations were found causing amino acid substitutions in exon 3 (patients P9-P10 and P16) or exon 4 (P14-P15). The missense mutation observed in P16 was located within the cytidine deaminase catalytic region in exon

# Non consanguineous families



# Consanguineous families

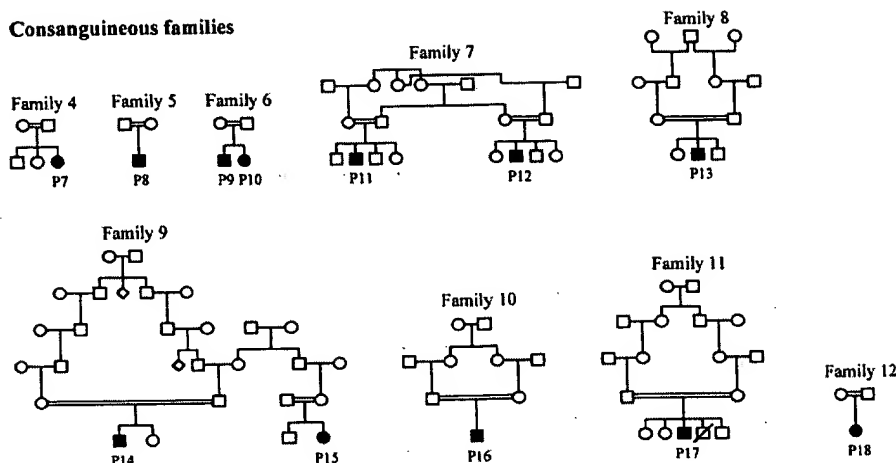


Figure 1. Pedigree of the HIGM2 Families

The figure depicts the pedigrees of the 12 families included in the study (9 consanguineous).

3 (Figures 2B–2D). A homozygous 19 bp deletion was observed in patient P17 at the beginning of exon 2, causing a frameshift and a premature stop codon. In the unrelated patients P6 and P8, an identical homozygous base change leads to a stop codon in exon 4. In all these families, parents were heterozygous for the mutation. In family 1 (P1–P2–P3), a single homozygous nucleotide

substitution was detected leading to a stop codon in the cytidine deaminase catalytic region in exon 3. The mutation was inherited from the father, who was heterozygous, but was not detected in the mother. This observation suggested a genomic deletion spanning exon 3 in the mother, confirmed by Southern blot analysis in the mother and affected children (data not shown). The

Table 1. Patients' Characteristics

| Family               | Origin  | Patients | Age at diagnosis (year) | Recurrent infections | Lymphoid hyperplasia | Ig levels (g/l) |       |         | IgM Antibody production                    |        |
|----------------------|---------|----------|-------------------------|----------------------|----------------------|-----------------|-------|---------|--|--------|
|                      |         |          |                         |                      |                      | IgM             | IgG   | IgA     | Hemagglutinins (titer × 10 <sup>-1</sup> ) | Anti-B |
| 1                    | Morocco | P1       | 6                       | +                    | +                    | 4.5             | <0.06 | <0.07   | 4  | 4      |
|                      |         | P2       | 5                       | +/-                  | +                    | 1               | <0.06 | <0.07   | 32   | 16     |
|                      |         | P3       | 1                       | +                    | +                    | 1               | <0.06 | <0.07   | NE   | 4      |
| 2                    | Morocco | P4       | 5                       | +                    | +                    | 2.4             | 0.4   | <0.07   | NE   | 64     |
|                      |         | P5       | 1                       | +/-                  | -                    | 1.5             | <0.06 | <0.07   | NE   | 64     |
| 3                    | Italy   | P6       | 6                       | +                    | -                    | 8               | <0.06 | <0.07   | 16   | 8      |
| 4                    | Turkey  | P7       | 2                       | +                    | -                    | 1.6             | 0.5   | <0.07   | ND   | ND     |
| 5                    | Italy   | P8       | 2                       | +                    | +                    | 9               | 0.3   | <0.07   | 32   | 4      |
| 6                    | Turkey  | P9       | 12                      | +                    | +                    | 10              | 0.5   | <0.02   | 16   | 16     |
|                      |         | P10      | 5                       | +                    | +                    | 11              | 0.1   | <0.02   | NE   | 2      |
| 7                    | Turkey  | P11      | 13                      | +                    | +                    | 7               | <0.06 | <0.02   | 512  | 6      |
|                      |         | P12      | 13                      | +                    | +                    | 17              | <0.05 | <0.02   | NE   | NE     |
| 8                    | Turkey  | P13      | 8                       | +                    | +                    | 34              | 0.05  | <0.02   | 128  | 512    |
| 9                    | Turkey  | P14      | 3                       | +                    | +                    | 9               | 0.7   | 0.1     | 1,024                                      | 512    |
|                      |         | P15      | 8                       | +                    | +                    | 11              | <0.02 | <0.02   | NE   | 128    |
| 10                   | Turkey  | P16      | 1                       | +                    | -                    | 10              | 1.3   | 0.2     | NE   | NE     |
| 11                   | Turkey  | P17      | 12                      | +                    | +                    | 37              | <0.02 | <0.02   | 32   | 32     |
| 12                   | Turkey  | P18      | 2                       | +                    | -                    | 5.6             | <0.02 | <0.02   | ND   | ND     |
| Age-matched controls |         |          | 1–5                     |                      |                      | 0.5–1.1         | 5–12  | 0.3–1.3 | 4–16                                       | 4–16   |
|                      |         |          | 5–13                    |                      |                      | 0.6–1.7         | 7–15  | 0.8–2.3 | 16–128                                     | 16–128 |

ND = Not done.

NE = Not evaluable (because of blood group).

Table 2. Patients' B Cell Characteristics

| Patients | Percentage of CD19 <sup>+</sup> /PBMC* | Percentage of CD27 <sup>+</sup> /CD19 <sup>+</sup> cells | In vitro induced IgE production in presence of sCD40L + IL-4 (ng/ml)** |
|----------|--|--|--|
| P1       | 21                                     | 10   | <0.1   |
| P2       | 17                                     | 21   | <0.1   |
| P3       | 14                                     | ND   | <0.1   |
| P4       | 16                                     | 23   | <0.1   |
| P5       | 17                                     | 29   | <0.1   |
| P6       | 18                                     | ND   | ND   |
| P7       | 20                                     | ND   | 0.3  |
| P8       | 18                                     | 19   | ND   |
| P9       | 13                                     | 14   | <0.1   |
| P10      | 9                                      | 9  | <0.1   |
| P11      | 14                                     | 13   | 0.5  |
| P12      | 12                                     | 19   | ND   |
| P13      | 14                                     | 28   | 0.4  |
| P14      | 14                                     | 5  | 0.2  |
| P15      | 12                                     | 45   | 0.3  |
| P16      | 15                                     | 15   | 0.7  |
| P17      | 17                                     | 9  | 0.3  |
| P18      | 26                                     | 21   | 8-24   |
| Controls | 5-25                                   | 10-47  |  |

ND = Not determined.

\* All CD19 B cells also express CD40.

\*\* Production of IgE in unstimulated B cells from patients and controls was always lower than 0.5 ng/ml.

same nucleotide substitution leading to a stop codon in exon 3 was found in patients from family 2 (P4-P5), inherited from the mother. The same haplotype was common to families 1 and 2, both from Morocco. A further distinct heterozygous mutation, a 9 bp deletion, inherited from the father, and also located in the cytidine deaminase catalytic region, was found in these 2 patients (Figures 2B-2D).

In contrast, no mutations were found in the *huAID* sequence in 106 normal chromosomes from unrelated individuals.

#### Expression of *huAID* Gene

Expression of the *huAID* gene was investigated in tonsils from normal controls and patient P4 by RT-PCR and Northern blot analysis. *HuAID* mRNA was similarly detected by both methods in tonsil material from P4 and control (Figures 3A and 3B). This was not an unexpected finding, given at least one of P4 mutations (Figure 2C).

Because murine AID expression has been found in B cells induced to switch in vitro (Muramatsu et al., 1999), the expression of *huAID* gene in control human B cells was investigated following activation by a combination of sCD40L and IL-4. *HuAID* transcripts, which were not detectable at day 0, were detected by RT-PCR after a 5 day culture of PBMC from controls (Figure 3C) and remained detectable after 8 and 12 days of culture (data not shown). In the same culture conditions, *huAID* mRNA was either normally detected (patients P5, P6, and P17), or weakly detected (patients P1 and P3) (Figure 3C). The latter finding may be due to the likely instability of *huAID* mRNA carrying a stop codon in the first half of the molecule.

#### Ig V Gene Somatic Mutations in HIGM2 Patients

In order to investigate other possible consequences of *huAID* defect in B cell differentiation, the rate and pattern

of somatic mutations in the V3-23 Ig V gene, a member of the VH3 family expressed in 4%-10% of B cells (Stewart et al., 1993), was analyzed. In the first set of experiments, the frequency of mutation of V3-23-C $\mu$  transcripts expressed by CD19<sup>+</sup> blood B cells from patients P4 and P18 was respectively 0% with 13/13 sequences devoid of mutations (P4) and 0.4% mutation per bp with 5/9 sequences unmutated (P18). These results differed from those obtained with a normal adult control (2% mutation per bp) and a 2 year old control (2.5% mutation per bp).

In a second set of experiments, the mutation frequency of the V3-23-C $\mu$  transcripts expressed by purified CD19<sup>+</sup> CD27<sup>+</sup> B cells from these two patients and three other patients were analyzed. The frequency of V3-23 mutations from patients was significantly reduced and ranged from 0% to 0.9% mutation per bp, as compared to 2.6% to 6.3% mutation per bp in 3 different age-matched controls (Table 3). Of note, in patients P2 and P17, all individual clones sequenced were completely devoid of mutation. The pattern of mutations accumulated in the V3-23 gene in control B cells, unlike the rare mutations found in patients' B cells, showed the major individual hotspots of mutations in CDRs previously reported (Levy et al., 1998) and reflects the process of antigenic selection. These results clearly show that the hypermutation process is profoundly defective in AID-deficient B cells.

#### Germinal Center B Cells in Lymphoid Organs of HIGM2 Patients

Histological sections of enlarged tonsils from patients P1, P4, and P8 and cervical lymph nodes from patient P4 showed identical marked follicular hyperplasia. GC were 5 to more than 10 times larger than GC from control reactive lymph nodes (Figure 4A). Mantle zone and inter-follicular areas were present. Mantle zone B cell phenotype was normal (i.e., IgM<sup>+</sup>, IgD<sup>+</sup>, CD23<sup>+</sup>, CD38<sup>-</sup>,

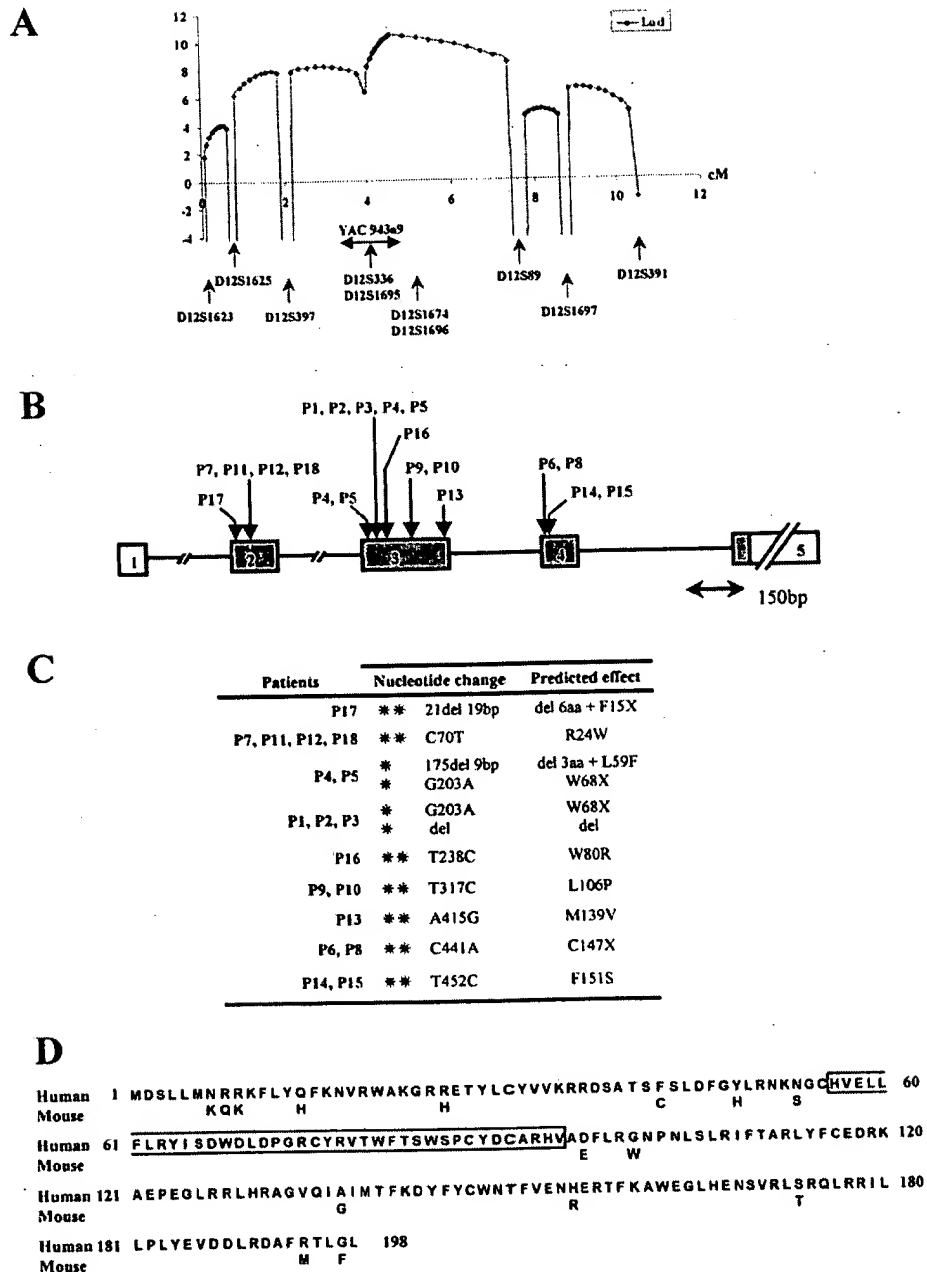


Figure 2. Localization and Genetic Analysis of huAID Mutations in HIGM2 Patients

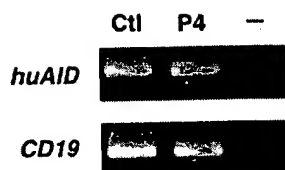
(A) Multipoint LOD score analysis. The telomeric end of chromosome 12 is located on the left. A maximum LOD score of 10.45 was obtained between polymorphic markers *D12S397* and *D12S89*. Among the YAC contig, the 943a9 YAC was shown to harbor markers *D12S336* and *D12S1695* and *huAID* exon 2.

(B) Localization of mutations in the *huAID* gene. Nine different mutations were found in the coding sequence (red) in 18 patients tested. Mutations in patients P1-P2-P3, P4-P5, and P16 were localized in the cytidine deaminase catalytic region. In patients P1-P2-P3, a further heterozygous deletion was detected by Southern blot (not shown).

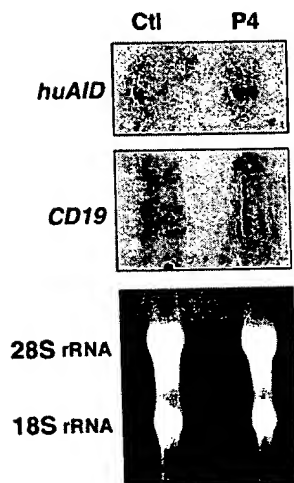
(C) Mutations in the *huAID* gene. In 10 out of 12 families, the mutations were homozygous in patients (\*\*), and in two families, heterozygous mutations were found (\*). Among these mutations, 7 were nucleotide substitutions causing missense mutations in 5 cases, or a stop codon (X) in 2 others. Two other changes were nucleotide deletions leading to one missense mutation and one stop codon.

(D) Alignment of the human and mouse amino acid sequences. The conserved cytidine deaminase motif is indicated by an open box. Only nonidentical residues are indicated for mouse.

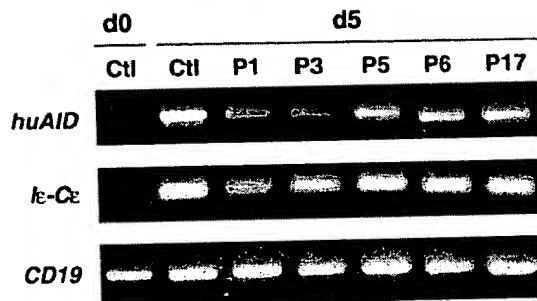
A



B



C

Figure 3. Expression of *huAID* Transcripts

(A) *HuAID* transcripts were analyzed by RT-PCR in tonsils from a control (Ctl) and patient P4 as compared with *CD19* transcripts. (-), no cDNA.

(B) Northern blot analysis: similar amounts of *huAID* mRNA were found in patient and control tonsils, although more *CD19* mRNA was present in the patient, in accordance with the increased number of B cells detected by immunohistology. Loading control is shown by BET gel staining (lower panel).

(C) *huAID*, *CD19*, and *Iε-Cε* transcripts were detected by RT-PCR on RNA isolated from PBMC before (d0) in control (Ctl), or after 5 day stimulation (d5) by sCD40L and IL-4 in control (Ctl) and patients. The *HuAID* RT-PCR product found in patient P6 was smaller due to an exon "skipping", leading to a deletion of 35 bp, which includes the stop codon detected on the genomic DNA and lets the transcript out of frame.

*Iε-Cε* sterile transcripts were normally amplified in patients, although no IgE production was obtained after 12 days (see also Table 2). As a control, *CD19* transcripts were similarly amplified.

Table 3. Mutations in V3-23-C $\mu$  Transcript Sequences from *CD19*<sup>+</sup>*CD27*<sup>+</sup> Purified B Cells

|                    | Clones |            | Mutations |           |
|--------------------|--------|------------|-----------|-----------|
|                    | All    | Unmutated* | Total     | Per bp, % |
| 2 yr old control   | 10     | 0          | 172       | 5.9       |
| 3.5 yr old control | 7      | 2          | 52        | 2.6       |
| 11 yr old control  | 10     | 1          | 182       | 6.3       |
| P2                 | 9      | 9          | 0         | 0         |
| P4                 | 8      | 7          | 3         | 0.1       |
| P8                 | 10     | 9          | 22        | 0.76      |
| P17                | 12     | 12         | 0         | 0         |
| P18                | 9      | 3          | 24        | 0.9       |

\* Unmutated =  $\leq 1$  mutation.

*Bcl-2*<sup>+</sup>, and *Ki67*<sup>-</sup>) (Figure 4A, arrows, and data not shown). Giant GC contained a normal follicular dendritic cell network (*DRC*<sup>+</sup>, data not shown), and *PNA*<sup>+</sup>, *CD38*<sup>+</sup>, *CD23*<sup>+</sup>, *CD83*<sup>+</sup>, *CD95*<sup>+</sup>, *CD40*<sup>+</sup>, *IgM*<sup>+</sup>, *Bcl2*<sup>-</sup>, and *Ki67*<sup>+</sup> B cells (Figures 4A and 4B and data not shown). Strikingly, numerous GC B cells coexpressed *slgD* (Figures 4A and 4B), contrasting with observations in normal reactive GC in which GC *IgD*<sup>+</sup> cells are scarcely found (Figure 4A). The high proliferation frequency of B cells in GC was associated with a dense network of macrophages filled with apoptotic bodies (Figure 4B, open arrows) that gave the GC a starry sky appearance. A dark zone and a lighter zone could be distinguished in some patients' follicles on *Ki67* staining (data not shown). However this "light zone" also contained numerous cycling cells and *slgD*<sup>+</sup> cells. The phenotype and size of the GC B cells identify them as proliferating (*Ki67*<sup>+</sup>) germinal founder cells (*CD38*<sup>+</sup>, *slgM*<sup>+</sup>, and *slgD*<sup>+</sup>) as described by Lebecque et al. (1997). *SlgD*<sup>+</sup> and *slgM*<sup>+</sup> cells were also numerous in T cell areas (Figure 4A). Occasional *CD27*<sup>+</sup> *CD3*<sup>-</sup> cells as well as *IgM* and *IgD* plasma cells were found in GC and T cell areas. Neither *IgG* nor *IgA* plasma cells were observed (data not shown).

## Discussion

### *HuAID* Gene Mutations Cause HIGM2 Syndrome

In this study, compelling evidence is provided that mutations in the coding sequence of the *huAID* gene are responsible for the HIGM2 syndrome. We studied 18 HIGM2 patients from 12 families who fulfilled the diagnostic criteria of HIGM2. Genetic studies clearly demonstrated a strong linkage between the HIGM2 locus and a short genetic region on chromosome 12p13, which contains the human counterpart of mouse *AID* (Muto et al., 2000). Sequencing of the coding sequence of *huAID* revealed deleterious mutations in all 12 families, all along the gene. Widely scattered point mutations in *huAID* are all defective, indicating the strong structural constraint of *AID* in agreement with its evolutionary conservation between mouse (Muramatsu et al., 1999) and human (Muto et al., 2000). Some of the mutations are predicted to lead to truncated *huAID* proteins by generating stop codons or deletions. Several of the missense mutations were localized within the putative cytidine deaminase catalytic region of the protein (Muto et al., 2000). Despite the lack of an accessible experimental model to validate the effects of the *huAID* gene mutations, the association of the HIGM2 phenotype with *huAID* gene mutations together with the similar phenotype observed in *AID*<sup>-/-</sup>

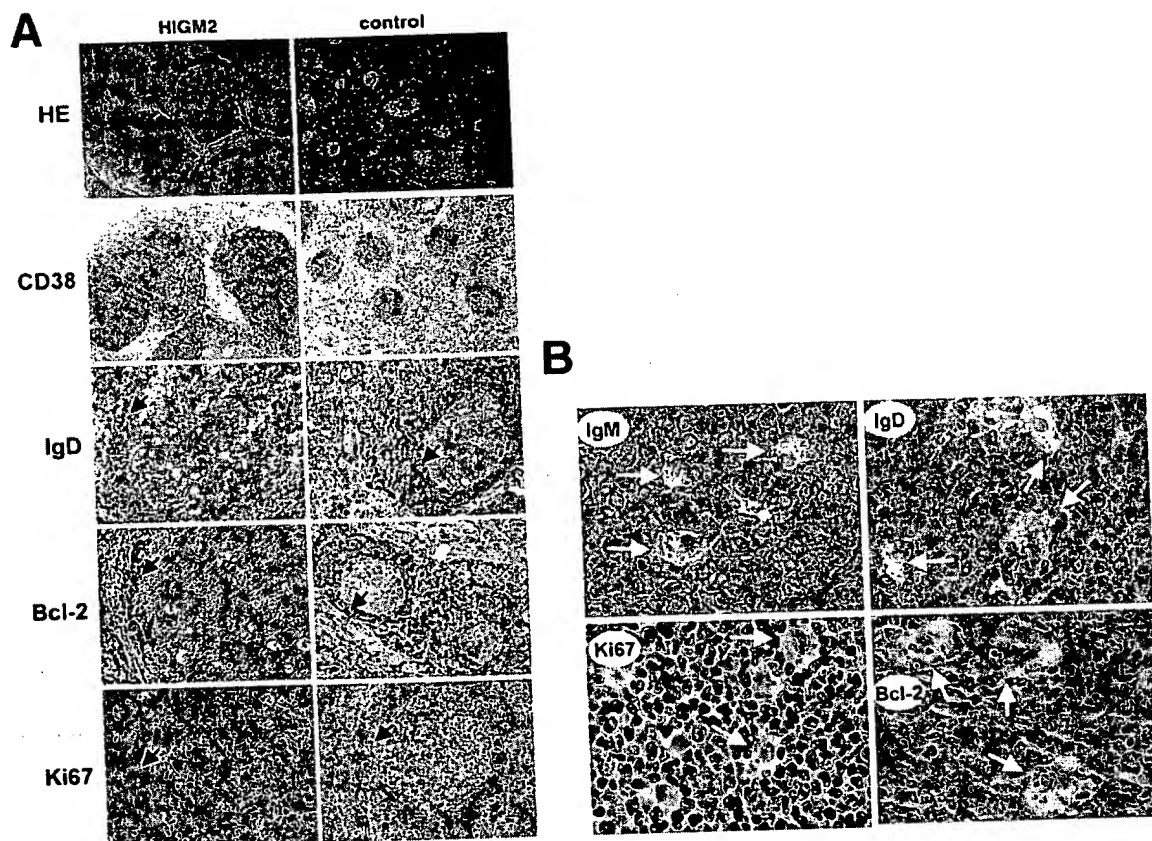


Figure 4. Immunohistological Examination of Cervical Lymph Node from Patient P4

(A) Comparison between histology and immunophenotype of P4 cervical lymph node and a control reactive lymph node. On hematoxylin-eosin staining (magnification  $\times 25$ ), P4 lymph node shows follicular hyperplasia with giant GC, compared with classical follicular hyperplasia, as control, shown at the same magnification. GC B cells from patient and control express CD38 (blue staining, original magnification  $\times 50$ ). Anti-IgD Ab stained numerous cells in interfollicular areas, as well as mantle cells (arrow), and most of the GC B cells from the patient, but not of control (brown staining, original magnification  $\times 100$ ). Bcl-2 staining indicated that most GC cells did not express Bcl-2, in contrast to the mantle zone and interfollicular areas, a pattern similar to that observed in control (brown staining, original magnification  $\times 100$ ). Ki67 staining reveals that most of the GC B cells from the patient are cycling (brown staining, original magnification  $\times 100$ ). An identical picture was observed in 2 other patients.

(B) Expression of IgM, IgD, Ki67, and Bcl-2 (brown staining) at higher magnification ( $\times 400$ ) in a HIGM2 germinal center from P4. On comparable sections, the large majority of GC B cells coexpress IgD, but only occasional small lymphocytes—presumably T cells—express Bcl-2. Note the very numerous macrophages filled with apoptotic bodies (open arrows). An identical picture was observed in 2 other patients.

mice (Muramatsu et al., 2000 [this issue of *Cell*]) demonstrates the causative role of *AID* gene mutations in the disease. The phenotype observed in this group of patients was fairly homogeneous and consisted of 3 abnormal features characteristic of the disease, i.e., (1) defective CSR, (2) defective Ig variable region gene somatic mutation generation, and (3) finding of enlarged GC with highly proliferating B cells.

#### Role of *huAID* in CSR

HIGM2 is characterized by absent or very low serum IgG and IgA while B cells are unable to undergo Ig switch in vitro (Callard et al., 1994; Conley et al., 1994; Durandy et al., 1997), indicating that *AID* is involved in CSR. However, the CSR mechanism is complex and not completely elucidated. CSR requires germline transcription (Gu et al., 1993; Jung et al., 1993; Xu et al., 1993; Zhang et al., 1993; Bottaro et al., 1994), an efficient splicing

machinery (Lorenz et al., 1995; Hein et al., 1998), and the formation of RNA:DNA complexes with R-loop structure at the S regions (Daniels and Lieber, 1995; Tracy and Lieber, 2000). The role of DNA-PK and the Ku70-Ku80 recombination complex in CSR has also been clearly demonstrated (Rolink et al., 1996; Casellas et al., 1998; Manis et al., 1998), whereas RAG molecules are not required (Lansford et al., 1998). It is likely that *AID* plays a role downstream of the germline transcription since we observed normal induction of sterile  $I\epsilon$ -C $\epsilon$  transcripts by CD40 agonists and IL-4 in HIGM2 patients. In addition, activation-induced de novo protein synthesis is required to perform CSR (Muramatsu et al., 1999), and the existence of a CSR recombinase has been postulated (Kinoshita and Honjo, 2000). Thus *AID* could be, if not the CSR recombinase itself, at least an essential part of the CSR complex machinery (Muramatsu et al., 1999).

### Role of *huAID* in Ig Variable Region Gene Somatic Hypermutations

Peripheral B cells from HIGM2 patients, though they normally express CD27, carry very rare Ig V gene somatic mutations compared with normal controls, based on the study of V3-23 genes in 5 cases showing that AID is also involved in the generation of Ig V gene somatic mutations. The precise mechanism responsible for somatic mutations is not well understood. The Ig genes have to be transcriptionally active to undergo somatic mutations (Peters and Storb, 1996; Fukita et al., 1998). It has been suggested that the DNA mismatch repair system, as well as a newly described DNA polymerase ( $\mu$ ) are involved in somatic mutation generation (Casalho et al., 1998; Wiesendanger et al., 1998; Dominguez et al., 2000).

The role of AID in the generation of somatic hypermutations is also unknown. Since CSR and somatic mutation processes can occur independently (Karttinen et al., 1983; Jacob and Kelsoe, 1992; Liu et al., 1996), it is unlikely that defective somatic mutation generation prevents subsequent CSR in HIGM2 patients. *HuAID* could rather act, as an RNA-editing enzyme, on one or several templates generating yet unidentified factor(s) involved in CSR and/or somatic mutation generation (Muramatsu et al., 2000).

A residual level of CSR was found in a few patients (for instance, patients P7, P14, and P16). Several hypotheses can account for this finding, i.e., residual AID activity associated with missense mutations or a role for AID-independent factor(s) as suggested in family 9 (patients P14-P15) since no CSR could be detected in P15. Persistence of maternal B cells due to fetal tolerance might be one possible factor. Interestingly, detection of residual CSR did not correlate with occurrence of somatic mutations as observed in patient P18. While no serum IgG and IgA were detectable in this case, a low but significant frequency of somatic mutations was found. Genetic or environmental factors may generate some variability as serum IgG was detectable in patient P7 but not in P18, who carries the same *AID* mutation. However, one can suggest that an AID protein carrying a missense mutation (R24W) could still be active in one pathway, thus implying that targets of AID in CSR and somatic hypermutation differ.

### Role of *huAID* in Controlling GC B Cell Proliferation

In sharp contrast to the lack of germinal centers (GC) in HIGM1 patients (Facchetti et al., 1995), lymphadenopathy and tonsillar hypertrophy were frequently observed in HIGM2 patients. Histological examination revealed giant GC filled with highly proliferating B cells. The presence within GC of numerous macrophages filled with apoptotic bodies indicate that intense apoptosis also occurs. Therefore, *huAID* function is not involved in either T cell-mediated nor BCR-mediated signaling events required for B cell proliferation or apoptosis (Garrone et al., 1995; Lagresle et al., 1996; Rathmell et al., 1996). Strikingly, GC proliferating B cells in HIGM2 patients express IgM, IgD, and CD38. This phenotype has previously been described on a small B cell subset corresponding to germinal center founder cells (Lebecque et al., 1997). Normally, these cells are thought to represent a transitional stage between follicular mantle and GC B cells, at the onset of Ig variable region gene somatic mutation and antigen-driven selection (Lebecque et al.,

1997). One possible explanation for our observation is that, in absence of functional AID, B cells are continuously triggered to proliferate by antigen (as they normally express the BCR), as long as no successful Ig variable region gene somatic mutations have occurred.

### Concluding Remarks

The observed consequences of AID deficiency in humans together with the phenotype described in the *AID*<sup>-/-</sup> mouse (Muramatsu et al., 2000 [this issue of *Cell*]) clearly demonstrate a crucial role of AID in Ig switch, Ig variable region gene somatic mutation generation, and normal germinal center formation. *HuAID* function, although unidentified, appears to be a key element controlling the steps required for terminal B cell differentiation. These data will enable further molecular delineation of the mechanisms required for B cell terminal maturation and efficient antibody responses.

### Experimental Procedures

#### Immunological Study of B Cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation. Immunofluorescent studies were performed by using specific antibodies: FITC or PE anti-CD19 monoclonal antibodies (mAb), PE-anti-CD27 mAb from Immunotech (Marseille, France), FITC-anti-CD40 mAb from Diaclone (Besançon, France), PE-goat anti-hu IgM Ab, and FITC-goat anti-hu IgD Ab from Caltag, (San Francisco, CA). Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) (Durandy et al., 1990). In vitro IgE switch was assessed in cultures of PBMC stimulated with soluble CD40L, 1  $\mu$ g/ml (Morris et al., 1999) (a kind gift from Immunex, Seattle, WA) and IL-4 (100 U/ml, R&D system, Minneapolis, MN). IgE concentrations were evaluated in supernatants by ELISA at day 12 (Durandy et al., 1997).

#### Linkage Analysis

Genomic DNA was extracted from peripheral blood leukocytes with proteinase K, sodium dodecyl sulfate, and a series of phenol-chloroform extractions. Microsatellite markers were studied using radio-labeled primers from the Genethon collection. The FASTLINK 2.2 package was used for genetic linkage analysis (Cottingham et al., 1993) and the GENEHUNTER 1.1 program was used for multipoint linkage analysis (Kruglyak et al., 1995). A YAC contig was obtained from CEPH, Paris, France (Krauter et al., 1995).

#### *HuAID* Gene Sequencing

The five exons of *huAID* were amplified by polymerase chain reaction (PCR) using Thermal Cycler 9700 from Perkin Elmer (Norwalk, CT), specific primers, and Taq High Fidelity (Roche Diagnostic, Mannheim, Germany).

Primers for exon 1: 5'-CATTAAATTGAAGTGAGATTTTCTGG-3' (forward) 5'-AGC ATTTGTGTGGAAACTCTGG-3' (reverse) (1 cycle at 94°C 1 min, then 40 cycles at 94°C 30 s, 54°C 1 min, 68°C 6 min). Primers for exon 2: 5'-GAGTTTGAGGTACAAGTTGGACAC-3' (forward) 5'-ACAAGC TGATAATATTCTCCCCAT-3' (reverse) (1 cycle at 94°C 1 min, then 40 cycles at 94°C 30 s, 54°C 30 s, 72°C 1 min). Primers for exons 3, 4, 5: 5'-TATCTCCTCTCTCTAACACGCT-3' (forward) 5'-GATA CTCTCATTAGGAGGTCC-3' (reverse) (1 cycle at 94°C 1 min then 40 cycles at 94°C, 30 s, 54°C 1 min, 72°C 3 min).

PCR products were separated by electrophoresis and purified (Qiagen GmbH, Hilden, Germany). The 5 exons and adjacent intronic regions were sequenced with the dRhodamine dye terminator cycle sequencing kit (ABI prism) and analyzed with the ABI prism 377 genetic analyzer from Perkin Elmer.

#### *HuAID* Gene Expression Study

Total RNA from tonsillar tissue, unstimulated PBMC, or PBMC stimulated for 5–12 days with sCD40L (1  $\mu$ g/ml) and IL-4 (100 U/ml) was extracted by disruption in guanidium thiocyanate followed by ultracentrifugation through a cesium chloride cushion. Pelleted RNA was



extracted with phenol chloroform and precipitated with ethanol. Single-strand cDNA transcription was produced with 5 µg of total RNA, oligodT and reverse transcriptase (Superscript II, from Gibco Life technologies, Paisley, Scotland).

RT-PCR was performed with primers for AID: 5'-GAGGCAAGAAG AACTCTGG-3' (forward) and 5'-GTGACATTCCTGGAAGTTGC-3' (reverse) (1 cycle at 94°C 5 min, then 35 cycles at 94°C 1 min; 56°C 1 min., 72°C 2 min).

Amplification of CD19 and *Iε-Cε* transcripts was performed (primers available if requested).

For Northern blot analysis, 10 µg of total mRNA from tonsils was electrophoresed on alkaline agarose gels and transferred to charge nylon membranes overnight. Northern blots were hybridized to the radio-labeled *huAID* probe, then stripped and rehybridized to the radio-labeled CD19 probe as control. Radioactivity was determined by using a phosphorimager.

#### Cloning and Sequencing of V3-23-C $\mu$ Transcripts

CD19<sup>+</sup>/CD27<sup>+</sup> and CD19<sup>+</sup>/CD27<sup>-</sup> B cells were sorted using a FACSTAR PLUS (Becton-Dickinson). The purity of CD19<sup>+</sup>/CD27<sup>+</sup> B cells was greater than 99% and contamination by CD27<sup>+</sup> cells in the CD19<sup>+</sup>/CD27<sup>-</sup> population was less than 1%. Total RNA extraction and cDNA synthesis was performed as previously described (Levy et al., 1998) with the following modifications: C $\mu$  A (5'-GAGGCAGCT CAGCAATC-3') primer was used for cDNA synthesis. PCR was performed with 0.5 U of Pfu polymerase (Stratagene) and the primers: V3-23 leader exon (5'-GGCTGAGCTGGCTTTTCTTGTGG-3') and C $\mu$  B (5'-TCACAGGAGACGAGGGGAA-3') (35 cycles at 94°C 45 s, 60°C 1.5 min; 72°C 2 min). PCR products were cloned using the TA cloning kit (Invitrogen) and V3-23 positive colonies were sequenced with the dRhodamine dye terminator cycle sequencing kit (ABI prism) and analyzed with the ABI prism 310 genetic analyzer.

#### Immunopathology of Lymph Nodes and Tonsils

Immune reactive tonsils from patients P1, P4, and P8 and enlarged cervical lymph node from P4 were surgically removed for diagnostic purposes. Paraffin embedded sections were stained with hematein-eosin and serial cryostat, or, paraffin sections were incubated with PNA (Vector Laboratories, Burlingame, CA) or mouse IgG1 antibodies to IgM (R1/69), IgD (IgD26), Bcl-2 (124), Ki67 (MiB1 and Ki67), CD3 (UCHT1), mouse IgG2a antibodies to CD20 (L26) from DAKO, Glostrup, Denmark; mouse IgM anti follicular dendritic cells (DRC, R4/23), and mouse IgG1 antibodies to CD38 (T16) from Immunotech (Marseille, France) followed by a goat anti-mouse alkaline phosphatase-conjugated or peroxidase-conjugated antibody. Fast blue (Sigma) and DAB (Sigma) were used as substrates for alkaline phosphatase and peroxidase, respectively (Cordell et al., 1984).

#### Acknowledgments

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, Assistance Publique/Hôpitaux de Paris (PHRC: AOM 96104), Association Française contre les myopathies, European Biomed2 Program (PL 963007), Italian Telethon (grant E.668 to LDN), and Immune Deficiency Foundation (grant to L. D. N.). P. R. was supported by a doctoral fellowship from Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, and A.G. was supported by a PPP Healthcare Medical Trust Mid-Career Award. We thank Drs. M. Debré and A. Deville, who take care of some of the patients. We acknowledge Pr. F. Facchetti, Drs. F. Le Deist, F. Rieux-Laucat, and J. P. de Villartay for helpful discussions, Mrs. F. Seltz for cell sorting experiments and Mrs. Tiouri for excellent secretarial assistance. We also thank Dr. Morris from Immunex for the kind gift of soluble CD40-ligand.

Received May 9, 2000; revised June 15, 2000.

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#### GenBank Accession Numbers

The genomic and cDNA sequences of *AID* reported in this paper have been deposited in GenBank with accession numbers AB040430 and AB040431, respectively.